Novel Quinic Acid Derivative KZ-41 Prevents Retinal Endothelial Cell Apoptosis Without Inhibiting Retinoblastoma Cell Death Through p38 Signaling

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PURPOSE. To determine whether a novel NF-κB inhibitor, KZ-41, can inhibit melphalan’s actions on retinal endothelial cell (REC) inflammation and apoptosis, without eliminating the chemotherapeutic efficacy of melphalan on cell death of retinoblastoma cells (Y79).

METHODS. RECs were cultured in M131 medium supplemented with growth factors and antibiotics. Once cells reached confluence, they were treated with or without 10 μM KZ-41, following treatment with 4 μg/mL melphalan. Cell proteins were extracted and analyzed for intracellular adhesion molecule 1 (ICAM-1) levels and Cell Death ELISA. RECs were also transfected with or without NF-xB siRNA or treated with SB202190 (p38 [mitogen activated protein kinase] MAPK inhibitor) before melphalan treatment to determine the involvement of NF-xB and p38 MAPK in REC apoptosis and ICAM-1 levels. We also cultured retinoblastoma cells (Y79) in RPMI-1640 medium supplemented with 20% fetal bovine serum and performed a Cell Death ELISA after melphalan + KZ-41 treatment to determine if the treatments altered melphalan’s ability to promote cell death of Y79 cells.

RESULTS. KZ-41 inhibited melphalan-stimulation of ICAM-1 levels and REC apoptosis, whereas KZ-41 did not alter melphalan’s effects on Y79 cells. KZ-41’s protective effects on REC were mediated through p38 MAPK activation. Although KZ-41 blocked both NF-xB and p38 MAPK-dependent ICAM-1 stimulation; the p38 MAPK/ICAM-1 pathway appears to be the primary pathway involved in melphalan-induced REC apoptosis.

CONCLUSIONS. KZ-41 protects REC against melphalan-induced upregulation of ICAM-1 and apoptosis through p38 MAPK-dependent pathways.

Keywords: KZ-41, NF-xB, Y79, retinal endothelial cell, cell death, inflammation

Retinoblastoma is the most common primary intraocular malignancy in children. With treatment, greater than 90% of patients in developed countries survive. However, to improve the quality of life of these cancer survivors, newer treatments using localized chemotherapy have been developed in hopes of better salvaging eyes and vision. Selective ophthalmic artery chemotherapy was first developed by Kaneko in the 1980s using a balloon occlusion of the carotid artery to selectively deliver chemotherapy to the ophthalmic artery.1 Subsequently, Abramson modified Kaneko’s approach by directly delivering melphalan, a nitrogen mustard, into the ophthalmic artery under manual control without balloon occlusion, a technique termed super-selective intraophthalmic artery chemotherapy (SSIOAC).2,3 While initial results suggested that SSIOAC was more effective than systemic chemotherapy for specific cases, recent data indicate that a number of significant side effects to the retinal and choroidal vasculature can occur following SSIOAC with melphalan.4–6 For example, melphalan SSIOAC administered to nonhuman primates (NHPs), in the same manner as given to children, produced retinal vascular inflammation manifested as increased leukocyte adhesion and occlusion.7

To better understand the characteristics and possible causes of SSIOAC-induced vascular toxicities, we used in vitro cultures of human retinal endothelial cells (RECs) to assess direct toxic effects of melphalan. We found that exposure to a retinoblastoma cell-cidal melphalan dose (4 μg/mL)7 produced a greater than 6-fold increase in REC death, which was associated with a significant increase in both mRNA and protein levels of key inflammatory mediators, specifically interleukin-8 (IL-8) and intracellular adhesion molecule 1 (ICAM-1).

Since ICAM-1 appeared to be linked to REC apoptosis, we wanted to determine the cellular signaling from melphalan to ICAM-1 and subsequently to apoptosis. In the experiments reported here, we tested three candidate mediators (TNF-α, NF-xB, and p38 [mitogen activated protein kinase] MAPK) to determine which of these might trigger an increase in ICAM-1 levels that specifically leads to REC apoptosis. It has been
reported that TNF-α can regulate ICAM-1 in human dermal microvascular cells.9 In addition, both TNF-α and NF-κB can regulate ICAM-1 levels and are associated with apoptosis.9,10 In human umbilical vein endothelial cells, Yu et al. found that another chemotherapy agent, cisplatin, increased ICAM-1 levels using NF-κB-dependent pathways.10 Finally, it has been reported that p38 MAPK also regulates ICAM-1 expression in human umbilical vein endothelial11 and in primary glial cell cultures.12 p38 MAPK has also been shown to regulate apoptosis in T lymphocyte Jurkat cells.13 A novel agent, KZ-41, has been recently reported to decrease TNF-α and NF-κB activity14,15 and therefore holds some promise as a protective treatment against TNF-α and NF-κB-mediated ICAM-1 stimulation and apoptosis. The effects of KZ-41 on p38 MAPK have not previously been reported. Our experiments described herein test our hypothesis that melphalan induces REC apoptosis through an ICAM-1 pathway triggered by increases in TNF-α, NF-κB, and/or p38 MAPK, and furthermore, that KZ-41 offers significant protection against melphalan’s REC effects. We also include initial experiments to explore the potential for using KZ-41 as a protective adjunct therapy for SSIOAC. In order to be considered for treatment, KZ-41’s effects would ideally be limited to REC and it should not block melphalan’s desired, proapoptotic effects on retinoblastoma tumor cells.

METHODS

Reagents
Melpalan was bought from Bioniche Pharma (Lake Forest, IL). KZ-41 was supplied by C. Ryan Yates, PhD, PharmD. An ICAM-1 ELISA kit was purchased from Millipore (Billerica, MA). An Cell Death ELISA kit was purchased from Roche Applied Science (Indianapolis, IN). NF-κB, phospho-NF-κB (S536), p38 MAPK, phospho-p38 MAPK (T180/Y182) antibodies, and SB202190 (10 μM, p38 MAPK inhibitor, blocks p38α and p38β) were purchased from Cell Signaling (Lake Placid, NY). ICAM-1 antibody was purchased from R&D Systems (Minneapolis, MN). Actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A transfection reagent was purchased from Invitrogen (Lipofectamine RNAiMAX, Carlsbad, CA). Human Scr sRNA (ON-TARGET plus nontargeting Pool D-001810-10); human ICAM-1 sRNA (ON-TARGET plus SMARTPool L-003502-00-0005); human TNF-α sRNA (ON-TARGET plus SMARTPool L-010546-00-0005); and human NF-κB ssiRNA (ON-TARGET plus SMARTPool L-003520-00-0005) were purchased from Dharmacon RNAi Technologies (Chicago, IL). Etanercept was obtained from Arnold Postlewaite, PhD. Secondary anti-mouse and anti-rabbit antibodies conjugated with horseradish peroxidase were purchased from Promega (Madison, WI). ECL for immunoblot development and signal detection was purchased from Amersham Biosciences (Piscataway, NJ).

Cell Culture
RECs were provided by Cell System Corporation (Kirkland, WA) and grown in Medium 131 containing microvascular growth supplements (MVGS), 10 μg/mL gentamycin, and 0.25 μg/mL amphotericin B. Cultures were maintained at 37°C in a humidified 95% air and 5% CO2 atmosphere. Only primary cells within passage 6 were used. RECs were growth-arrested by incubating in Medium 131 for 24 hours and used to perform the experiments unless otherwise indicated. The use of growth-arrested cells is key since we are studying many pathways activated by growth factors. We commonly use this practice in our laboratory.16,17

Y79 retinoblastoma cells were purchased from ATCC (Manassas, VA). Cells were grown in suspension in RPMI medium with antibiotics and 20% fetal bovine serum. Cells were starved overnight before any treatments.

Cell Death Assay
An equal number of RECs were placed into the 96-well plates and cultured to 90% confluence. Cells were starved without growth factor overnight and treated with the drug for 24 hours. Cells were washed with PBS twice and resuspended in 200 μL lysis buffer, then incubated for 30 minutes at room temperature. Lysates were centrifuged at ×200g for 10 minutes, and 20 μL of cell lysates were transferred into the streptavidin-coated microplate under gentle shaking for 2 hours at 20°C. Supernatants were removed and the wells were washed with incubation buffer. 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt solution was added to develop color detected at 405 nm (vs. 490 nm reference).

ICAM-1 ELISA
An ELISA for ICAM-1 level was performed using an ICAM-1 ELISA assay kit according to the manufacturer’s instructions (Millipore) to evaluate the ICAM-1 level following treatment with melphalan and KZ-41. ICAM-1 siRNA, TNF-α siRNA, NF-κB siRNA, or etanercept (10 μM). For all ELISA analyses, equal protein amounts were loaded into each well.

Western Blotting
After appropriate treatments and rinsing with cold phosphate-buffered saline, RECs were lysed in the lysis buffer containing the protease and phosphatase inhibitors and scraped into tubes. Equal amounts of protein from the cells were separated on the precast tris-glycine gel (Invitrogen), then blotted onto a nitrocellulose membrane. After blocking in tris-buffered saline with Tween 20 (10 mM Tris–HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) bovine serum albumin, the membranes were treated with NF-κB, phospho–NF-κB, p38 MAPK, phospho–p38 MAPK or ICAM-1 antibodies (1:500) followed by incubation with horseradish peroxidase-labeled secondary antibodies. The antigen-antibody complexes were detected using a chemiluminescence reagent kit (Pierce ECL Western Blotting Substrate—ECL—enhanced chemiluminescence; Thermo Scientific, Waltham, MA).

Transfections
RECs were transfected with ICAM-1 siRNA, TNF-α siRNA, or NF-κB siRNA at a final concentration of 20 nM using a transfection reagent (Invitrogen) according to the manufacturer’s instructions. After transfection, cells were starved in MVGS-free Medium 131 for 24 hours and used as required.

Statistics
All the experiments were repeated in triplicate, and the data are presented as mean ± SEM. Data was analyzed by Kruskal–Wallis nonparametric test followed by Dunn’s test with P values < 0.05 considered statistically significant. In the case of Western blotting, one representative blot is shown.
RESULTS

**KZ-41 Inhibited Melphalan-Induced REC Apoptosis Without Affecting Apoptosis of Y79 Retinoblastoma Cells**

We have previously published that 4 µg/mL melphalan increases REC cell death.7 We repeated that experiment to verify that 4 µg/mL does increase DNA fragmentation. To eliminate the adverse effects on REC, we used a novel agent, KZ-41, that is expected to reduce apoptosis, perhaps by blocking increases in NF-kB. The results showed that KZ-41 inhibits melphalan-induced REC apoptosis dose dependently, with a significant decrease at 10 µM (Fig. 1A). While it is important to demonstrate the KZ-41 inhibits melphalan-induced REC apoptosis, it is equally important to show that KZ-41 does not inhibit the death of retinoblastoma cells. We tested various doses of melphalan on Y79 retinoblastoma cells and found 4 µg/mL produced maximal apoptosis (Fig. 1B). KZ-41 did not affect apoptosis of the Y79 cells (Fig. 1C).

**KZ-41 Inhibited Melphalan-Induced ICAM-1 Levels in REC**

We have previously reported that melphalan increases ICAM-1 mRNA and protein levels.7 Since KZ-41 was effective in reducing apoptosis of REC, we also wanted to determine if KZ-41 could block the melphalan-induced ICAM-1 increase in REC. We performed an ICAM-1 ELISA after KZ-41 + melphalan treatment in REC. As shown in Figure 2, when 10 µM KZ-41 was added 30 minutes before melphalan treatment, REC significantly decreased ICAM-1 levels. Similar results were obtained when we performed a Western blot for ICAM-1 levels, demonstrating that the ICAM-1 ELISA and Western blot produce similar results.

**Melphalan-Induced Increases in ICAM-1 Levels in REC Do Not Involve TNF-α**

Since there is an abundance of literature showing that TNF-α can regulate ICAM-1,7,8,18 we first wanted to measure...
whether inhibition of TNF-α using TNF-α siRNA or etanercept, a TNF-α receptor antagonist, could reduce melphalan-induced increased ICAM-1 levels and REC apoptosis. Neither TNF-α siRNA nor etanercept-reduced ICAM-1 levels after melphalan treatment. (C) Cell Death ELISA results for REC treated with etanercept and/or melphalan. While etanercept did reduce melphalan-induced REC apoptosis, it did not reach statistical significance. *P < 0.05 versus control. #P < 0.05 versus melphalan only. Data are mean ± SEM. n = 4.

Melphalan-Induced ICAM-1 Activation Involves NF-κB and This Pathway Is Inhibited by KZ-41

To understand the mechanism by which melphalan caused increased ICAM-1 in REC, we first tested whether NF-κB is involved. Melphalan (4 μg/mL) induced NF-κB phosphorylation in a time-dependent manner in REC. Maximum increases in NF-κBSer536 phosphorylation occurred at 30 minutes; increased levels

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**FIGURE 3.** The role of TNFα in the regulation of ICAM-1. ICAM-1 ELISA results for REC treated with sc siRNA, TNF-α siRNA (A), or etanercept (B) and melphalan. Neither TNF-α siRNA nor etanercept-reduced ICAM-1 levels after melphalan treatment. (C) Cell Death ELISA results for REC treated with etanercept and/or melphalan. While etanercept did reduce melphalan-induced REC apoptosis, it did not reach statistical significance. *P < 0.05 versus control. #P < 0.05 versus melphalan only. Data are mean ± SEM. n = 4.

**FIGURE 4.** The role of NF-κB in the regulation of ICAM-1. (A) Western blot results for the ratio of phospho-NF-κB to total NF-κB after melphalan treatment. (B) Western blot results for ratio of phospho-NF-κB to total NF-κB after melphalan and KZ-41 treatments. (C) A control experiment to demonstrate that NF-κB siRNA was able to significantly reduce NF-κB protein levels. (D) ICAM-1 ELISA results after melphalan and NF-κB siRNA treatment. *P < 0.05 versus control. #P < 0.05 versus melphalan only. Data are mean ± SEM. n = 4 for all experiments.
of NF-kB levels were maintained for at least 2 hours (Fig. 4A). In addition, melphalan-induced ICAM-1 upregulation was blocked by either KZ-41 (Fig. 5B) or NF-kB siRNA (Fig. 4C). Figure 4D demonstrates that NF-kB siRNA significantly reduces NF-kB levels.

**Melphalan-Induced ICAM-1 Activation Also Involves p38 MAPK and This Pathway Is Also Inhibited by KZ-41**

To determine whether p38 MAPK is involved in melphalan-induced ICAM-1 levels, we first wanted to determine whether melphalan significantly increased phosphorylation of p38 MAPK. We showed that melphalan does increase phosphorylation of p38 MAPK, which was blocked by KZ-41 (Fig. 5A). Next, we tested whether SB202190 (a p38 inhibitor) altered ICAM-1 levels by ELISA. The results showed that when p38 MAPK was blocked with the SB202190 inhibitor, KZ-41 was ineffective in inhibiting melphalan-induced upregulation of ICAM-1 levels (Fig. 5B).

**Melphalan-Induced REC Apoptosis Is Mediated Through p38 MAPK, but Not NF-kB; This Apoptotic Pathway Is Inhibited by KZ-41**

While we demonstrated above that KZ-41 blocks ICAM-1 stimulation by both NF-kB and p38 MAPK pathways, the important experiment is to determine which of these pathways are key in triggering REC apoptosis mediated by melphalan. Our results (Fig. 6) show that the p38 MAPK inhibitor, SB202190, and ICAM-1 siRNA were able to block melphalan-induced REC apoptosis, whereas NF-kB siRNA did not alter REC apoptosis (Fig. 6D). These results suggest that while melphalan stimulates both the p38 MAPK/ICAM-1 pathway and the NF-kB/ICAM-1 pathway, these pathways have divergent effects. Furthermore, the p38 MAPK/ICAM-1 pathway is the primary trigger for REC apoptosis.

**DISCUSSION**

Super selective intraophthalmic artery chemotherapy using melphalan continues to be used for the treatment of retinoblastoma, despite the reports of deleterious changes to the retina and choroid of children. While a number of potential factors may be at play in producing these negative side effects—such as the pH of melphalan (pH 5.5), pulsatile flow, and particle formation—we propose that mitigating the direct melphalan-induced REC toxicity may provide the best option to improve this novel therapy. We have previously reported both increased ICAM-1 levels in human REC and retinal vascular toxicity in nonhuman primates treated with melphalan.

To address the increased ICAM-1 levels and apoptosis in REC, we attempted to find an agent that could protect REC, while maintaining melphalan-induced apoptotic effects in Y79 retinoblastoma cells. We identified KZ-41, a novel quinic acid derivative, as a potential candidate. KZ-41 is in preclinical development for the prevention and mitigation of injury from whole body irradiation exposure (Thompson et al., unpublished observations, 2011). Melphalan and irradiation similarly trigger p53 dependent apoptosis. Thus, we hypothesized that KZ-41 would protect REC from melphalan-induced apoptosis. Indeed, one of our major findings is that KZ-41 is effective in preventing melphalan-induced apoptosis in REC, without altering melphalan’s actions on retinoblastoma cells. Whether or not selective protection of normal tissue by KZ-41 extends to other genotoxic insults (e.g., irradiation) is being investigated.

Early work with KZ-41 demonstrated that it acts as an anti-inflammatory agent through regulation of TNF-α and NF-kB. Our first goal was to determine if TNF-α was involved in the regulation of ICAM-1 and REC apoptosis. As noted in Figure 4, neither TNF-α siRNA nor etanercept could prevent ICAM-1 levels; furthermore, etanercept did not prevent REC apoptosis. This finding was unexpected since the majority of literature supports TNF-α-mediated regulation of ICAM-1. Since TNF-α did not reduce ICAM-1 levels, we next investigated NF-kB, as TNF-α and NF-kB most often work in concert to regulate ICAM-1 and apoptosis. We found that melphalan was able to increase phosphorylation of NF-kB, which was attenuated with treatment with KZ-41 or use of NF-kB siRNA. While NF-kB does not stimulate ICAM-1 levels in REC cells, NF-kB siRNA did not protect against apoptosis. Additionally, our data show that ICAM-1 siRNA can protect against melphalan-induced apoptosis, further suggesting ICAM-1 and apoptosis are potentially linked in the REC. While NF-kB appears to stimulate ICAM-1, this regulation does not trigger melphalan-induced apoptosis in REC. Our findings may be similar to what
was observed after radiation exposure to p53 null cell lines (Saos-2). In order to determine other pathways involved in both ICAM-1 and apoptosis, we initiated studies of p38 MAPK. Recent work with KZ-41 has suggested that it can regulate p38 MAPK in a model of radiation injury (Thompson, unpublished observations, 2011). We first show that melphalan increases the phosphorylation of p38 MAPK, which was inhibited by KZ-41 (Fig. 5). Secondly, we found that both KZ-41 and a p38 MAPK inhibitor blocked melphalan actions on ICAM-1 (Fig. 5), as well as inhibit DNA fragmentation (Fig. 6). p38 MAPK has been reported to regulate ICAM-1 expression in human umbilical vein endothelial cells at the level of gene transcription. This increase in ICAM-1 expression was reduced when SB203580 was used to inhibit p38 MAPK. Similarly, work in primary glial cell cultures demonstrated that p38 MAPK regulates ICAM-1. Activation of ICAM-1 by p38 MAPK in turn activated inflammatory cytokines. In addition to a role in the regulation of ICAM-1, p38 MAPK has also been reported to regulate apoptosis. Inhibition of p38 MAPK through SB202190 treatment increased apoptosis in T lymphocyte Jurkat cells. Our results suggest that while melphalan stimulation of the NF-kB pathway and the p38 MAPK pathway both lead to increased levels of ICAM-1, it is primarily the latter pathway that triggers REC apoptosis (Fig. 6).

Taken together, our data strongly suggest that melphalan induces REC apoptosis through altered p38 MAPK levels. ICAM-1 levels are highly involved in REC apoptosis, as ICAM-1 siRNA significantly reduced melphalan-induced REC apoptosis. We currently do not know why melphalan triggers p38 MAPK-dependent increases in ICAM-1 that lead to REC apoptosis, but similarly triggered NF-kB-dependent increases in ICAM-1 are not proapoptotic. Future work to dissect out pathways by which ICAM-1 mediates REC apoptosis and how p38 MAPK is involved may help to resolve this issue. In summary, we provide evidence that KZ-41 offers protection to REC against melphalan-induced retinal damage and, as such, KZ-41 is a promising drug candidate for providing a novel adjunct treatment for SSIOAC.

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